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(57) Abstract

A precursor polypeptide having the amino acid sequence: met-gln-arg-pro-phe-leu-leu-ala-tyr-leu-val-leu-ser-leu-leu-phe-asn-ser-ala-leu-gly-X, wherein X is a polypeptide. The precursor polypeptide, when produced in a eukaryotic host cell by the expression of a gene coding for the precursor polypeptide, is exported from the host cell and processed to produce the mature polypetide, X. The polypeptide may be an anzyme, a lymphokine, a hormone or the like. The yeast enzyme AMG, and the mammalian polypeptides interferon- 2 and gastric lipase are specifically exemplified.

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PRECURSOR POLYPEPTIDE, DNA SEQUENCE CODING THEREFOR, VECTORS, HOST ORGANISMS, AND PROCESSES INVOLVING SAME

Field of the Invention

This invention relates to the field of recombinant DNA biotechnology.

Background to the Invention

It has been suggested that starch (or dextrin)-degrading yeasts could be usefully exploited in fermentation processes, such as the production of ethanol or beer, which utilise starch-containing raw materials (Tubb, 1983, 1984: full reference to be found at the end of this specification), and in the production of amylolytic enzymes such as amyloglucosidases (Eveleigh, 1981; Fogarty, 1983). Dex^+ strains of Saccharomyces cerevisiae (formerly called S. diastaticus; van der Walt, 1970; Yarrow, 1984) have at least one DEX or STA gene (Erratt and Stewart , 1978; Tamaki, 1978) and are able to ferment soluble starch or dextrins as a consequence of producing an extracellular amylo-α-1,4-glucosidase (AMG) during vegetative growth (Hopkins, 1955; Searle and Tubb, 1981). However, common brewing strains of Saccharomyces cerevisiae do not have the ability to produce extracellular AMG during vegetative growth. Dex^+ strains of Saccharomyces cerevisiae have been hybridised with brewing strains, and progeny have been derived which have the ability to produce AMG during vegetative growth. However these progeny strains produce unacceptable low carbohydrate beers (Tubb et al, 1981), unless steps are taken to eliminate a gene (POF1) responsible for a 'herbal phenolic' off-flavour (Goodey and Tubb, 1982).

Recombinant DNA techniques offer a more specific approach to conferring amylolytic character on strains of yeast already possessing many other desirable commercial characteristics. Recently, α-amylase genes from mice (Thomsen, 1983) and wheat (Rothstein et al, 1984) have

been expressed in S.cerevisiae, and a gene for AMG production has been cloned from a STA1 strain of yeast (Yamashita and Fukui, 1983).

A 3.6kb DNA fragment has been cloned from a Saccharomyces diastaticus genome (strain BRG536): DEX1) and shown to confer production of extracellular amylo-α-1, 4-glucosidase (AMG), and thereby, the ability to hydrolyse starch and dextrins, on Dex$^-$ strains of S. cerevisiae (Tubb, R.S., Brewers Guardian, Sept. 1984, 34-37). (A preliminary report of this work was given at the ALKO Symposium on gene expression in yeast at Helsinki in June, 1983).

The use of a eukaryotic signal sequence to promote product transport from a eukaryotic host cell harbouring a recombinant vector is known. Published European patent application EP-A1-0127304 describes fusion polypeptides comprising a signal (or "pre") sequence and a desired polypeptide produced by expression of a gene in a host cell. The fusion polypeptides are transported through the host cell membrane and cleaved to produce extracellular, mature, polypeptides. The yeast invertase signal sequence is specifically mentioned and its use in the preparation of host cells capable of producing extracellular interferon is described. Published European patent application EP-A1-0116201 describes an essentially similar use of eukaryotic signal sequences and exemplifies the use of the yeast α-factor signal sequence in the production of extracellular human epidermal growth factor (hEGF). The existence and use of the signal sequence of a yeast amylolytic enzyme to promote product secretion of heterologous polypeptides from yeast has been the subject of speculation (Tubb, R.S, Brewers Guardian, Sept. 1984, 34-37)

The cloned 3.6kb DNA fragment referred to above has now been sequenced and the coding sequence for AMG

has been identified and elucidated. In addition, it has been unequivocally shown that the AMG gene includes a leader sequence coding for a signal peptide capable of promoting product secretion.

According to the present invention, there is provided a precursor polypeptide having the amino acid sequence:

met-gln-arg-pro-phe-leu-leu-ala-tyr-leu-
val-leu-ser-leu-leu-phe-asn-ser-ala-leu-gly-X

wherein X is a polypeptide.

The precursor polypeptide, when produced in a eukaryotic host cell by the expression of a gene coding for the precursor polypeptide, is exported from the host cell and processed to produce the mature polypeptide,X.

The polypeptide X may be any polypeptide such as an enzyme, a hormone, a lymphokine or the like. The mature polypeptide may itself be a precursor such as a pro form. Particular examples described herein are the yeast enzyme AMG, the mammalian enzyme, gastric lipase and the mammalian lymphokine, interferon-α2. The amino acid sequences for AMG and human gastric lipase are shown in Figures 4 and 5 respectively.

In a further aspect of the invention there is provided a DNA sequence coding for a precursor polypeptide according to the invention. The DNA sequence may be used to construct vectors for use in transforming eukaryotic host cells. The DNA sequence may have the following nucleotide sequence:

5' -ATGCAAGACCATTTCTACTCGCTTAT-
TTGGTCCTTTCGCTTCTATTTAAC-
TCAGCTTTGGGT(X^1)-3'

wherein X^1 is the coding sequence of the polypeptide X.

In a further aspect of the invention, there is provided a eukaryotic expression vector including a DNA sequence of the invention positioned relative to a

promoter capable of directing expression of the DNA sequence when the vector is transformed into a eukaryotic host cell, provided that when the polypeptide X is amylo-α-1, 4-glucosidase, the promoter is not an amylo-α-1, 4-glucosidase gene promoter.

The promoter may be any functionally active eukaryotic promoter but is preferably a yeast promoter, such as a promoter derived from the phosphoglycerate kinase (PGK) gene. The vector is adapted for expression in a eukaryotic host cell by the provision of selectable markers and control regions as, appropriate.

In a further aspect of the invention there is provided a eukaryotic host organism transformed with a vector of the invention. The host organism may be any eukaryotic organism including mammalian cells in tissue culture, but is preferably a yeast. Especially preferred are strains of Saccharomyces cerevisiae. Where the polypeptide X is an amylolytic enzyme, the yeast is preferably a brewing strain.

In a further aspect of the invention there is provided a fermentation process for producing ethanol comprising the step of culturing a yeast, in the presence of starch or dextrin, transformed with a vector of the invention including a gene coding for an amylolytic enzyme.

Such a fermentation will allow for the super-attenuation of wort liquor and consequently the production of a low-carbohydrate beer.

In a further aspect of the invention there is provided a process for the production of a polypeptide comprising growing, in a culture medium, a eukaryotic host organism transformed with a vector of the invention and isolating the polypeptide from the culture medium.

The invention is now described with reference to the following Examples which refer to the accompanying drawings in which:

Figure 1 - shows plasmid and restriction maps of plasmids pLHD30 and pLHD301,

Figure 2 - shows a comparison between strains of extracellular proteins after elution from DEAE-Sepharose,

Figure 3 - shows the effect of temperature (Figure 3a) and pH (Figure 3b) on the activity of AMG from BRG 536(O) and from AH22[pLAD30](□),

Figure 4 - shows the complete DNA and amino acid sequence of the AMG precursor, The reading frame for the AMG amino acid sequence is the top-most of the three reading frames commencing, for the AMG precursor polypeptide, with the methionine residue (M) corresponding to the ATG codon at nucleotides 144-146 and ending with the TAG stop codon at nucleotides 2562-2564. The mature AMG amino acid sequence commences with the phenylalanine residue (F) corresponding to the TTT codon at nucleotides 207-209.

Figure 5 - shows the complete DNA and amino acid sequence of human pregastric lipase,

Figure 6 - shows a restriction map of plasmid pYC3,

Figure 7 - a photograph (7(a)) and an autoradiogram (7(b)) at an agar plate, including colonies of HGL secreting transformed yeast hosts,

Figure 8 - shows a Western blot polyacrylamide gel of the protein products of a transformed yeast, and

Figure 9 - shows a restriction map of plasmids pMA91-DEX, pMA301-1 and pMA91-DEX-IFN.

In the DNA sequence of Figures 4 and 5 and elsewhere in the present description:

G - denotes a guanosine nucleotide residue
T - denotes a thymidine nucleotide residue
A - denotes a adenosine nucleotide residue, and
C - denotes a cytosine nucleotide residue

In the amino acid sequences of Figures 4 and 5 and elsewhere in the present description:

A - denotes an alanine residue
C - denotes a cysteine residue
D - denotes an aspartic acid residue
H - denotes a histidine residue
E - denotes an glutamic acid residue
F - denotes a phenylalanine residue
G - denotes a glycine residue
H - denotes a histidine residue
I - denotes an isoleucine residue
K - denotes a lysine residue
L - denotes a leucine residue
M - denotes a methionine residue
N - denotes an asparagine residue
P - denotes a proline residue
Q - denotes a glutamine residue
R - denotes an arganine residue
S - denotes a serine residue
T - denotes a threonine residue
V - denotes a valine residue
W - denotes a tryptophan residue, and
Y - denotes a tyrosine residue

EXAMPLE 1

Materials and Methods

1) Strains and Plasmids

The yeast strains used are listed in Table 1 below. Escherichia coli DH1 (F⁻recA1 endA1 gyrA96 thi-1 hsdR17 supE44), was used for the propagation and amplification of plasmid DNA. The yeast-E.coli vector was that denoted pJDB207 (Beggs, 1981).

TABLE 1

Strain	Known genotype[a]	Origin or derivation
X4003-5B	a leu2 ade1 his4 met2 ura3 gal1 cdx1	Genetic Stock Centre, Berkeley
AH22	a leu2 his4 CDX1	Hinnen et al (1978)
BRG536	α DEX1 cdx1	Goodey and Tubb (1982)
BRG136B	a lys2 DEX1 CDX1	(b)
BRG205B	a ade1 DEX1 CDX1	(c)
BRG136D	a dex1	(b)
BRG139A	a dex1	(b)
BRG140A	a dex1	(b)

[a]Since a Cdx^+ or Cdx^- phenotype can only be observed in Dex^+ strains, the genotype of Dex^- strains (X4003-5B and AH22) were deduced by analysis of the meiotic progeny from crosses with DEX1 CDX1 strains.

[b]Derived from a cross between BRG536 and SA (Goodey and Bevan, 1983)

[c]Derived from a cross between BRG536 and CB11 (ten Berge et al, 1973).

2) Yeast Growth Media

The medium denoted 'YPG' contained 20g glucose, 10g yeast extract and 10 g bacto-peptone per litre. In the medium denoted 'YP', glucose was omitted and in the medium denoted 'YP5G', the glucose concentration was

increased to 50g l^{-1}. The medium denoted 'GYNB' contained 20g glucose and 6.7g Yeast Nitrogen Base ('YNB'; Difco) per litre. In the medium denoted 'SGYNB'. the glucose concentration was increased to 30g l^{-1} and the medium (pH 5.4) was buffered with 0.05 M citrate (12.7 mM citric acid, 37.3 mM sodium citrate). The medium denoted 'DEXYNB' contained dextrin (Sigma, Type IV; 20g l^{-1}) instead of glucose. Auxotrophic requirements were met by additional supplements (100µg ml^{-1}) where necessary. In order to impose a selection for maintenance of plasmids, Leu^+ transformants growing in YNB media were not provided with leucine. Where required, media were solidified with agar (20g l^{-1}). Sorbitol (1.2 M) was incorporated as osmotic stabiliser when regenerating transformants.

3) Isolation, Restriction, Ligation and Analysis of DNA

Plasmid DNA was prepared from yeast by the method of Brown et al (1981). Total DNA was prepared in the same way, but the centrifugation step immediately following cell lysis was omitted. DNA for cloning was purified further by caesium chloride (CsCl) density gradient centrifugation.

Bulk and mini-scale preparations of plasmid DNA from E.coli were performed by alkaline lysis (Maniatis et al 1982). Horizontal gel electrophoresis was carried out as described by Brown et al (1981).

Restriction enzymes BamHI, EcoRI, HindIII and PstI, were purchased from Boehringer-Mannheim, BglII and KpnI from P + S Biochemicals, and Sau3A from Bethesda Research Laboratories. T4 DNA ligase was from New England Biolabs. Enzyme reactions (digestions and ligations) were carried out in accordance with the suppliers' instructions. For cloning experiments, linearised vector DNA was treated with alkaline phosphatase from calf intestine (Boehringer-Mannheim;

Bolivar and Backman, 1979) prior to ligation with genomic fragments.

4) Transformation Procedures

Yeast transformation was carried out using the procedure of Beggs (1978) except that double the concentration of plasmid DNA was used. Transformation of E.coli DH1 was performed following the method of Maniatis et al (1982) for E.coli X1776.

5) Southern Transfers and Hybridisations

Radiolabelled DNA was prepared from ^{32}P-labelled dCTP (Amersham International) using a nick translation kit (Bethesda Research Laboratories) in accordance with the manufacturer's instructions. Transfers and hybridisations (Southern, 1975) were carried out with Biodyne A nylon membranes (Pall Process Filtration Ltd.) using protocols provided by the manufacturer. Autoradiography was performed at room temperature using Fuji RX film with intensification.

6) Other Genetic Techniques

Mating, sporulation of diploids, analysis of meiotic progeny and other general genetic procedures were essentially as described by Sherman and Lawrence (1974). A Dex$^+$ phenotype was scored as growth on DEXYNE agar and confirmed by testing cells or culture supernatants (prepared as described in (7) below) for the presence of AMG by the rapid method of Searle and Tubb (1981).

7) AMG Activity Measurements

In examining the regulation of AMG production and the location of AMG activity, inocula were prepared by growing strains for 48h at 25^{o}C in GYNB and then in YP5G for a further 24h. Conditions of glucose excess were maintained by subculturing into YP5G (10 to 30µl into

10ml). Glucose-limited growth was established by using YP medium (100 ml) and a diffusion capsule (LH Engineering) containing 1ml glucose solution (50%, w/v). When growing transformants, loss of plasmid was monitored by plating out culture samples for single colonies on GYNB + leucine, replica plating to GYNB (without leucine) and determining the proportion of Leu^- colonies.

Culture supernants for assay were recovered by centrifugation. To prepare a cell homogenate, the yeast pellet was washed twice and suspended in its own weight of citrate-phosphate buffer (48.5 mM sodium citrate, 103 mM Na_2HPO_4, pH5). Cells were disrupted with glass beads in a Braun homogeniser cooled with liquid CO_2 and the cell homogenate was decanted from the glass beads. To prepare protoplasts, yeast cells were suspended in 0.86 M mercaptoethanol, 1.2 M sorbitol, 25 mM EDTA, pH8, for 20 minutes at 30°C, and then for 90 minutes in 1.2 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, pH5.8 (5 ml), containing Zymolyase 60,000 (Miles Laboratories; 4mg ml^{-1}). The complete removal of cell wall material was checked in each case by microscopic examination. After three washes in the same buffer, protoplasts were lysed by the addition of water.

For assay, culture supernants were membrane filtered, dialysed at 4°C against citrate-phosphate buffer (pH5; 4 x 2 litres) and diluted 1:1 with the same buffer containing maltotriose (2%, w/v). Cell homogenates and protoplast lysates were diluted with water as necessary and assayed similarly at pH5 against maltotriose (1%, w/v) as substrate. Reactions in screw-capped tubes at 25°C were termintaed by immersion in boiling water, membrane filtered where necessary (assays of cell homogenates or protoplast lysates), and the glucose released by AMG from maltotriose measured by an enzyme-linked colorimetric procedure (Glucoquant Test Kit; Boehringer). In the

samples analysed, ∝-glucosidase (maltase) activity (assayed using p-nitrophenyl-∝-D-glucoside as substrate) was insignificant; therefore, inactivation of this activity by subjecting protoplast lysates and cell homogenates to 55°C (Searle, 1982) was omitted. Enzyme activity of cultures was related to cell dry weight which was determined by a standard procedure (Anon, 1977).

To examine the effect of pH on activity of purified enzyme, samples in citrate-phosphate buffer (pH5) were dialysed against deionised water to remove salts and diluted with buffer containing maltotriose. Buffers with pH values in the range 3.0 to 7.0 were constructed by mixing the required volumes of 0.05 M sodium citrate and 0.1 M Na_2HPO_4. To determine the effects of temperature on AMG activity, samples in citrate-phosphate, pH5, were held at temperatures in the range 0 to 70°C for 1 hour, cooled and assayed against maltotriose.

8) Analysis of Extracellular Proteins and Purification of AMG

Yeast strains were propagated aerobically in SGYNB and subcultured into the same medium (2 litres) using an inoculum of 2.5 [g. wet wt.] l^{-1}. Cultures were stirred (160 rev. min^{-1}) under air for 3d at 25°C. Culture supernatants were recovered by centrifugation, clarified by filtration (0.45μm; Millipore), and concentrated to between 10 and 20ml by ultrafiltration through a Diaflo PM30 membrane (Amicon). After overnight dialysis against 4 x 2 litres phosphate buffer (0.5M; pH7.0) concentrates were made 4M with respect to urea and passed through a DEAE-Sepharose CL-6B ion-exchange column (Pharmacia) equilibrated with phosphate buffer. Elution was with a 0 to 1M NaCl gradient in the same buffer, monitoring absorbance continuously at 280 nm and collecting 4 ml fractions. Fractions were assayed for protein (Lowry et al. 1951) and AMG activity (see (7) above).

Protein preparations were examined by SDS-PAGE using the method of Laemmli (1970). Gels were loaded with between 10 and 50µg protein per channel and after electrophoresis, were stained for protein using Coomassie Blue R250 or, for carbohydrate, using Fuchsin-sulphate (Zacharius et al, 1969).

For further purification, fractions possessing AMG activity after ion-exchange chromatography were pooled, concentrated with Aquacide IIA (Calbiochem.), diluted in a small volume of 0.05 M imidazone-HCl buffer (pH7.4) containing 4M urea, and applied to a chromatofocussing column containing Polybuffer Exchanger PBE 94 (Pharmacia). Elution was with polybuffer 74 (Pharmacia) over the range pH7 to pH4. Fractions containing AMG were again pooled, concentrated with Aquacide, diluted with citrate-phosphate buffer, pH5 (1 to 2ml) and, using the same buffer for elution, subjected to chromatography through a Biogel P150 (Bio Rad) gel-permeation column. This final purification step also served to remove the ampholytes used in chromatofocussing.

9) DNA Sequence Determination

The nucleotide sequence of the cloned 3.6 Kb fragment was determined by the dideoxy method (Sanger et al, (1977) after subcloning restriction fragments from pLHD301 (see below) into M13 mp9 or mp10 (Amersham) and transformation into JM101 (Messing and Vieira (1982).

10) Protein Sequence Determination

Mature AMG protein was produced by S.cerevisiae transformed with a PGK-based plasmid (pMA91 - see published European Patent Application EPO 073635) containing a gene for AMG derived from pLHD301 (see below). Protein was purified as described above in (8) and terminal amino acid sequence was determined by

automated Edman degradation using a gas phase sequenator.

Results

a) Cloning of a DEX gene

Total DNA (50µg) prepared from S. cerevisiae strain BRG536 was partially digested with Sau3A to generate fragments with an average length of 5Kb. These fragments were ligated in 200µl to 10µg BamHI-digested pJDB207. E.coli strain DH1 was transformed with the ligated mixture to generate a "bank" of 5×10^4 ampicillin-resistant, tetracycline-sensitive clones. Plasmid DNA was prepared from this bank and used to transform S. cerevisiae strain X4003-5B to Leu^+. Regeneration agar containing ca. 7×10^4 Leu^+ transformants was homogenised with sterile water in a domestic blender and samples (0.5ml) were spread onto plates of DEXYNB agar. A few strong-growing colonies (ca. 10 to 20 per plate) were obtained after 7 to 9 days at 25°C and shown to retain a Dex^+ Leu^+ phenotype when subcultured, whilst remaining auxotrophic for adenine, histidine, methionine, uracil and tryptophan. Plasmid DNA prepared from one of these yeast transformants was used to obtain ampicillin-resistant transformants of DH1, from which miniscale preparations of DNA were digested with E.coRI and analysed by agarose gel electrophoresis. Plasmid pLHD30 (see Figure 1a), conferred both Leu^+ and Dex^+ phenotype on X4003-5B by transformation. Restriction analysis revealed pLHD30 to possess both a small (0.9Kb) and a large (3.6Kb) insert separated by vector sequences which are orientatd in tandem. Such a structure could have arisen during ligation or by intermolecular recombination in vivo. (In Figure 1, DNA from BRG536 and DNA from pJDB207 are represented by the filled and empty areas respectively. Bm = BamHI, Bg = BglII, E = EcoRI, H = HindIII, K = KpnI, P = PstI, Figures are in Kb).

pLHD301 (see Figure 1b) was isolated from DH1 after transormation with pLHD30 DNA that had been partially digested by PstI and religated to remove the small insert and one vector sequence. This plasmid retained the ability to confer a Dex^+ phenotype on Dex^- strains thus showing that a functional DEX gene had been cloned, and is included within a 3.6Kb insert, which possesses single sites for the restriction enzymes BamHI, BglII, EcoRI, HindIII, KpnI and PstI (see Figure 1c).

Three separate deletions (labelled A, B and C in Figure 1c) were made into the cloned fragment using restriction and ligation. Plasmids carrying either deletion A or B failed to confer a Dex^+ phenotype on yeast transformants, whereas delection C did not inactivate DEX. Therefore greater than 1.5Kb of the insert, extending from the left hand vector-insert junction (see Figure 1) to an undetermined point between the EcoRI and BglII sites, appears to be essential for DEX expression.

(b) Production of AMG activity by Dex^+ transformants

DEX1 strains carrying the CDX1 allele retain AMG activity within the cell wall under conditions of glucose excess, whereas those carrying cdx1 accumulate AMG in the extracellular medium independently of the availability of carbon source (Searle, 1982). Cdx^+ and Cdx^- phenotype are readily distinguished by assaying supernatants for AMG activity from mid-log cultures in which excess glucose (2.5% w/v) is still present in the culture medium. A comparison of the abilities of BRG536 (cdx1) and BRG205B (CDX1) to produce extracellular AMG, under both excess-glucose and limiting-glucose conditions, is given in Table II. With glucose-limited growth, similar amounts of AMG activity were accumulated by both strains. However, under excess glucose conditions, BRG536, but not BRG205B, accumulated extracellular enzyme activity.

The finding that strains X4003-5B and AH22 are genotypically cdx1 and CDX1 respectively (see Table 1) provided the opportunity to examine the effects of the CDX1 gene on production of AMG in Dex$^+$ transformants. Three transformants were examined and all produced more (up to 5X) AMG activity than BRG536. Data representative of those obtained are given in Table II. Surprisingly, the presence of the CDX1 allele did not block production of extracellular AMG by transformants of AH22 under conditions of glucose excess. Given that the cloned DEX gene is DEX1, this result means that either (i) amplification of DEX1 expression, by cloning the gene on a multicopy vector, overcomes the regulatory effect of CDX1 or (ii) regulatory or structural regions of DEX1 required for CDX1 regulation, have been excised during cloning.

Although regulation of AMG production by CDX1 was not apparent in Dex$^+$ transformants, these strains did show a substantial increase in specific yield of AMG activity when grown under glucose-limited conditions. It is not known whether this effect, which is also seen with BRG536, is a specific glucose effect, or a consequence of much slower growth rates under limiting-glucose conditions (Table II).

Previous studies on dextrin-fermenting strains have indicated that active AMG is not found intracytoplasmically but is distributed between the cell wall and the external medium (Searle and Tubb, 1981a; Searle, 1982). Therefore, it was of interest to examine the effect of amplified production of AMG on the distribution of activity in CDX1 and cdx1 transformants. From Table III, it can be seen that all three of the Dex$^+$ transformants examined released proportionally more of their AMG activity to the culture medium than BRG536. AH22 transformants, which carry CDX1 and therefore might be expected to retain more AMG activity in the cell wall,

were particularly adept in accumulating AMG extracellularly. In contrast to BRG536, some AMG activity was detectable in the "cytoplasmic" fractions from Dex$^+$ transformants (Table III).

c) Identification and characterisation of AMG produced by a Dex$^+$ transformant

The AMG produced by AH22 [transformed with plasmid pLHD30] with that of BRG536 (DEX1) were compared. Extracellular proteins were concentrated by ultrafiltration and analysed by DEAE-Sepharose ion-exchange chromatography and by SDS-PAGE. The bulk of AMG activity from both AH22 [pLHD30] and BRG536 coincided with the first protein peak eluted from the DEAE-Sepharose (see Figure 2 in which the solid line represents absorbance at 280nm and the broken line represents AMG activity). This peak (and associated activity) was absent in AH22 [pJDB207] which lacks the cloned DEX gene.

TABLE II

Production of Extracellular Amyloglucosidase by Strains of S. cerevisiae

Strain/Transformant[a]	AMG activity (nmol glucose from maltotriose [mg dry cell wt]$^{-1}$ min^{-1} in supernatants from culture with: Excess glucose[b]	Limiting glucose[c]
BRG536	0.29	2.1
BRG205B	0[d]	1.9
X4003-5B	0	0
AH22	0	0
X4003-5B [pJDB207]	0	0
AH22 [pJDB207]	0	0
X4003-5B [pLHD30]	0.74	2.6
AH22 [pLHD30]	1.4	7.3
AH22 [pLHD301]	1.6	6.8

[a]With all transformants, the proportion of plasmid-zero cells(Leu$^-$ segregants) was below 5% during growth on selective medium (GYNB, no leucine added). At assay, after growth in non-selective conditions for up to 20 generations, 90% or more of the population retained a Leu$^+$ phenotype.

[b]Cultures assayed after 18h growth at which time glucoe was still present in the culture medium (ca. 3%, w/v).

[c]Cultures assayed after ca. 40h growth, at which time the cell density was similar to that of the 18h excess glucose culture.

[d]0 indicates not detected (i.e. < 0.05 activity units).

TABLE III

Distribution of AMG Activity in Dex$^+$ strains[a]

Strain	Proportion of AMG (% of total)[b] present as:		
	Extracellular (culture supernatant)	Cellular (cell homogenate)	Cytoplasmic (protoplast lysate)[c]
X4003-5B [pLHD30]	66	34	11
AH22 [pLHD30]	79	21	4
AH22 [pLHD301]	79	21	5
BRG536	58	42	0

[a]Strains were grown under conditions of glucose excess and assayed for AMG activity after 18h (see Table II).
[b]Total activity was derived by summing the activities of the culture supernatant and the corresponding cell homogenate.
[c]Cell membrane fragments were retained in this fraction during assay.

Analysis by SDS-PAGE of pooled fractions containing AMG activity revealed a major high molecular weight band in both AH22 [pLHD30] and BRG536. This band, which was absent in equivalent fractions of AH22 [pJDB207], stained strongly for carbohydrate indicating that it was a glycoprotein. Confirmation that this band did indeed represent AMG was obtained by further purifying the preparation from AH22 [pLHD30]. After chromatofocussing and gel permeation chromatography, a 27-fold purification of AMG activity was achieved in a recovery of 11.5% of total initial activity. This preparation, which analysed for protein:carbohydrate in the ratio 1:2.2, was judged homogeneous by SDS-PAGE, giving only a single band. Since AMG from AH22 (pLHD30] eluted from Biogel P-150 just after the void volume, the molecular size of the enzyme is about 150Kd. The dex^+ transformant, AH22 [pLHD30], produces an AMG of similar molecular size to that produced by BRG536. Further similarities in the enzymes produced by the two strains were apparent when the effects of temperature and pH on AMG activity were examined (Figure 3a and 3b: see also Table IV). Both enzymes were heat stable up to ca. 45°, were inactivated in parallel at higher temperatures (Table IV) and showed the same optimum pH (5.0) for activity (Figure 3b). Initial rates of activity over the temperature range 0 to 70°C were similar, with a maximum at 55°C (Figure 3a). (□ = AH22 [pLHD30], purified AMG, 16.6 units activity [mg protein]$^{-1}$: ○ = BRG536, pooled fractions 22-28 after ion-exchange chromatography 5.2 units AMG activity [mg protein]$^{-1}$)

TABLE IV

Temperature inactivation of an amyloglucosidase from AH22 [pLHD30] and BRG536.

Temperature °C	% Initial activity remaining after 1h	
	AH22 [pLHD30]	BRG536
25	100	100
35	100	100
45	94	97
55	22	23
70	0	0

d) Use of DEX as a selectable marker in yeast transformation

Dex$^+$ transformants of X4003-5B and wild-type strains with plasmids pLHD30 and pLHD301 were obtained by allowing plasmid-treated sphaeroplasts to regenerate in a complete medium before imposing the selection for Dex $^+$ (Table V). To impose a selection for dex$^+$, 10^9 sphaeroplasts which had been treated with plasmid DNA, were inoculated into molten YPG (20ml) containing sorbitol. After 48h at 25°C regeneration agar containing 2 to 5 x 10^4 micro-colonies was blended with water and samples (10 x 0.5ml) spread onto DEXYNE agar. After 7-9 days Dex$^+$ colonies appeared, surrounded by zones of cross feeding: up to 10 colonies were isolated on each plate. No Dex$^+$ colonies were obtained in the absence of plasmid DNA. Using this procedure the overall yield of transformants was substantially reduced when compared with a selection for Leu$^+$ during regeneration (see results obtained with X4003-5B in Table V).

e) DNA sequence of cloned fragment and identification of amino acid sequence of AMG precursor, mature AMG and AMG leader sequence

The nucleotide sequence of cloned 3.6kb fragment from BRG 536 was determined and is shown for nucleotides 21 to 2773 in Figure 4. This sequence contains on open reading frame corresponding to the upper of the three amino acid sequences shown in Figure 4 and terminating with the TAG stop codon at nucleotides 2562-2564.

The N terminal amino acid sequence determined for mature AMG obtained from S. cerevisiae transformed with a plasmid (pMA91) containing the cloned fragment is identical with the sequence in this reading frame which commences with the phenylalanine corresponding to the TTT codon at nucleotides 207-209.

TABLE V

Transformation of S. cerevisiae strains using DEX plasmids

Recipient strain	Plasmid	Selection	Transformants recovered (colonies /µg DNA)
X4003-5B	pJDB207	Leu$^+$	ca. 10,000
X4003-5B	pLHD30	Leu$^+$	ca. 10,000
		Dex$^+$	860
X4003-5B	pLHD301	Leu$^+$	ca. 5,000
		Dex$^+$	750
BRG136D	pLHD30	Dex$^+$	1,100
	pLHD301	Dex$^+$	860
BRG139A	pLHD30	Dex$^+$	85
	pLHD301	Dex$^+$	65
BRG401A	pLHD30	Dex$^+$	540
	pLHD301	Dex$^+$	430

On the basis of the DNA sequence alone, however, it is not clear at which point the leader sequence for the AMG starts. There are two methionine residues (M) in this reading frame corresponding to the ATG codons at nucleotides 111-113 and 144-146 which precede this phenylalanine. In order to determine which of these methionine residues corresponds to the start of the AMG leader sequence, the inserted plasmid DNA from a pMA91 was cleaved at the unique Stu 1 site at nucleotide 116 and the resultant DNA reconstructed into pMA91, the resultant plasmid including the sequence from nucleotide 116 to nucleotide 2564 but lacking the ATG codon corresponding to nucleotides 111-113. This resultant plasmid was used to transform S. cerevisiae which was shown to produce AMG in the same way as S. cerevisiae transformed with previous plasmids containing nucleotides 111-113. This shows that the AMG leader sequence commences with the methionine residue corresponding to the ATG at nucleotides 144-146 and identifies the 22 amino acid sequence of the AMG leader peptide.

The experiments detailed above describe the cloning of a gene (DEX) which can be used to confer production of extracellular amylo-α-1, 4-glucosidase on Dex^- strains of Saccharomyces cerevisiae. The DEX gene is shown to provide a selectable marker for plasmid transfer into wild-type strains. The use of a DEX gene, either by itself or in conjunction with the use of plasmids carrying the yeast CUP1 gene as a selectable marker (Henderson, 1983), can now be evaluated in the construction of amylolytic strains of yeast for brewing.

Dex^+ transformants show amplification of AMG production, which is an expected consequence of cloning a gene on a multicopy vector (Lacroute et al, 1981). However, regulation of AMG production was observed when

transformants grown with excess glucose were compared with those grown under glucose limitation. The specific yield of enzyme activity was 3 to 7-fold higher under the latter conditions. This is consistent with the observation of Carlson and Botstein (1982) that glucose regulation of SUC2 is conserved when the gene is maintained on a multicopy plasmid.

In AH22 transformants under conditions of glucose excess, some influence of the CDX1 gene was expected on accumulation of AMG in the extracellular medium. However, release of enzyme was not blocked under these conditions and only a small proportion of total activity was retained within the cell wall. Since the possibility that Dex^+ transformants produce a substantially altered (e.g. smaller) AMG has been ruled out (see above), the lack of influence of CDX1 in this case suggests that its regulatory effect in DEX1 strains such as BRG205B, is exerted not at the level of the cell wall, but at an earlier stage in AMG synthesis. For example, if CDX1 confers a step-down in production of AMG-specific mRNA, the effect could be overcome either by gene amplification or by excision of regulatory non-coding regions during cloning.

Finally, a northern blot analysis of the RNA transcribed from strain AH22 ($LEU2^-$, $HIS3^-$) transformed with pLHD301 was performed. The RNA from pMA91-DEX, (see Figure 9 of the accompanying drawings) a plasmid in which the expression of the DEX gene is under the control of the efficient PGK promoter was compared to that from pLHD301. Both plasmids are present at about 100 copies per cell. (Plasmid pMA91 - also known as pM3013 - is described in published European patent application EP-A2-0073653). To construct pMA91-DEX. the StuI site, just downstream from the first identified ATG in the DEX open reading frame was converted to a BglII[site and the

fragment inserted into the unique BglII expression site in pMA91. pLHD301 has about 100bp of the 5' non-coding region of the DEX gene. Both plasmids were transformed into AH22 and grown on either a glucose or dextrin carbon source. The RNA from pMA91-DEX is about 30 fold more abundant than that from pLHD301 on the dextrin source and about 50 fold more when grown on glucose. Thus the 100bp of 5' non-coding region on the DEX gene is not a good promoter. The levels of RNA from pLHD301 in either a dextrin or a glucose carbon source are similar to the chromosomal PGK transcript, present as about 1% if mRNA transcribed from a single-copy gene. The fact that the RNA levels are similar regardless of the carbon source also suggests that the sequences which permit regulation of DEX transcription are not present in this plasmid. The RNA is of the expected size (2.5Kb) and is present as about 1% of the mRNA population in yeast (data not shown).

The position at which the RNA in pLHD301 initiates was mapped by primer extension using a synthetic oligonucleotide. The 20bp primer was holmogolous to a region just after the second ATG. The sizes of the extended products suggests that there are two start sites, 20 and 28bp upstream from the second ATG. Both RNAs initiated on an A. The positions of the start sites are located in favourable positions relative to the potential TATA box located about 70bp upstream from the second ATG. It is probable that the second ATG apparent in the DNA sequence is used as the translation initiation codon, since it is the first ATG present on the transcripts.

The DEX gene contains a yeast consensus splicing signal, TACTAAC about 1Kb from the ATG. There are no good matches to the consensus splice donor or acceptor sites. It was possible that the DEX gene contained an intron which would be removed by splicing the RNA transcript. An S_1 protection experiment, set up to test

this, suggested that the gene does not contain an intron. The protected fragment was about 2.5Kb. If the RNA was spliced then two protected fragments of 1.0Kb or less and 1.4Kb or less would be expected, since the TACTAAC sequence would be located in the intron. Even at high concentration of S_1 nuclease no smaller fragments could be detected. The control experiment in which a DNA molecule which differed from an RNA transcript by 5bp mismatch in the centre of the RNA shows that the S_1 nuclease recognises this mismatch. Therefore, the splicing of an intron from the DEX RNA would most likely be detected in this experiment.

The RNA transcribed from the DEX gene in pLHD301 is present at about 1% of mRNA in yeast and is not spliced. There are two RNA initiation sites located 20 and 28bp upstream from the second ATG (identified from the DNA sequence). The RNA start sites are preceded by a TATA box located 70bp upstream from the second ATG. This ATG is present at the start of a potential leader peptide of 20 amino acids. This sequence has been engineered into pMA91 to examine whether it can direct the secretion from yeast of a human gastric lipase or interferon gene.

It has been shown that a brewing strain of S.cerevisiae (NCYC1324), transformed with a vector including the DEX gene, and thus capable of producing extracellular AMG, when used in brewing trials, produces a beer with a lower specific gravity than would be expected with untransformed cells of the same strain of yeast

Example 2

To demonstrate that the DEX gene can be used to direct the secretion of heterologous proteins from S. cerevisiae, an in phase translational fusion was made between the 5' end of the DEX gene and the intact met-human gastric lipase (hGL) gene.

The cloning and expression of the hGL is described in copending International patent application PCT/GB 85/00364.

A gene encoding human gastric lipase was isolated from a cDNA clone bank made from mRNA prepared from a sample of human stomach tissue. Human gastric lipaseclones were identified by homology with a cDNA clone of rat lingual lipase previously obtained as described in published European patent application EP-A1-0131418. (The disclosures of which are incorporated herein by reference). A freshly obtained section of human stomach wall tissue approximately 2cm wide was stored in liquid nitrogen. The section contained complete mucosal, muscle and serosa layers. mRNA was prepared by guanidinium isothiocyanate extraction of the frozen ground complete tissue (Maniatis et al (1982) "Molecular Cloning - A Laboratory Manual". Cold Spring Harbor Laboratory). Polyadenylated RNA was isolated from this by oligo-dT cellulose chromatography (Harris, T.J.R. et al (1975) J. Gen. Virol 29 299-312).

The presence of an mRNA species encoding an acid stable lipase was suggested by Northern Blot analysis (Thomas, P.S. (1980) PNAS USA, 77 5201-5205). By this technique polyadenylated stomach RNA was separated on the basis of molecular weight by gel electrophoresis and probed with a cDNA clone of the rat lingual lipase gene labelled by nick translation (Rigby P.W.J. et al J. Mol. Biol. 113, 237-251). This labelled gene specifically hybridised with a mRNA species with an apparent size of approximately 1500 bases. This mRNA species was of a size capable of encoding a protein of the apparent size of human gastric lipase together with untranslated 5' and 3' sequences of such a message.

cDNA was prepared to the human stomach mRNA. First strands were synthesised by poly(dT) priming and

elongation by AMV reverse tr anscriptase (Retzel, E.F. et al (1980) Biochemistry 19 513-518). Second strands were synthesised by the action of RNase H, E.coli DNA polymerase I and E.coli DNA ligase as described (Gubler, V. and Hoffman, B. (1983) Gene 25 263-269). The double stranded cDNA was tailed at the 3' ends with poly(dT) (Villa-Komaroff et al (1978) PNAS USA, 75: 3727). Tailed fragments were annealed into pBR322 which had been cleaved and poly(dG) tailed at the PstI site. These hybrids were transformed into E.coli DH1 competent for transformation (Maniatis et al (1982) "Molecular Cloning - A Laboratory Manual". Cold Spring Harbor Laboratory). The transformants were screened by colony hybridisation on nitrocellulose filters (Hanahan, D. and Melson, M. (1980) Gene 10 63-67). The hybridisation probe was the DNA fragment containing the coding region for rat lingual lipase labelled by nick translation (Rigby, P.W.J. et al (1977) J. Mol. Biol. 113 237-252).

Putative human gastric lipase clones wee mapped for restriction endonuclease cleavage sites and subjected to DNA sequencing (Sanger, A.J.H. (1980) "Methods in Enzymology" Academic Press 65 560-580) using a synthetic single-stranded oligodeoxyribonucleotide primer which hybridised to a region just 3' to the cloned segment. Clones were shown to encode the lipase by sequence homology with the rat lingual lipase cDNA sequence and comparison of the predicted sequence from the cDNA clones with the N-terminal amino acid sequence of native human gastric lipase isolated from stomach aspirate Figure 5. One clone was identified (pGL17), approximately 1450bp long containing the entire coding sequence for the gastric prelipase. The 5' end of the clone was shown to be within 20 nucleotides of 5' terminal nucleotide of the message. This was demonstrated by the sequence obtained from the primer extension. In this technique a synthetic

oligodeoxyribonucleotide primer was hybridised specifically to a region of the human gastric lipase mRNA encoding the N-terminal protein sequence. This primer was extended to the 5' end of the mRNA and the sequence determined (Figure 5).

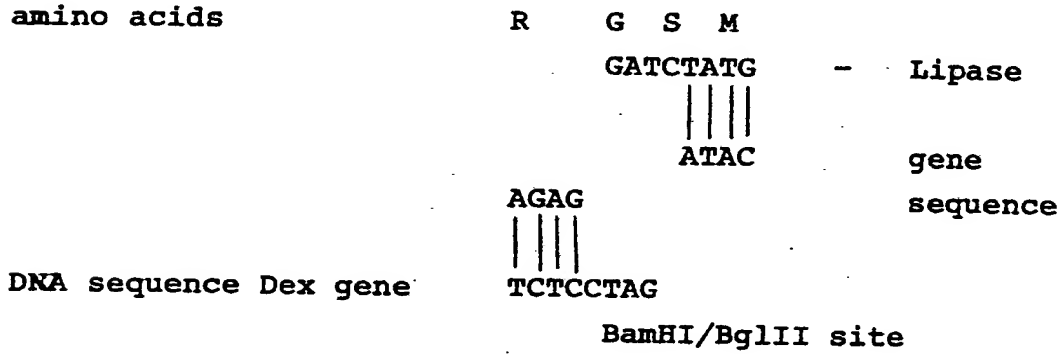
The DNA sequence of the coding strand of the pre human gastric lipase gene is shown in Figure 5. Numbers below the DNA sequence represent the base number. Base 1 is the first nucleotide of the cloned human gastric lipase sequence in pGL17. An "*" indicates the stop codon TAG which is followed by a 3' untranslated region. Underlined letters above the derived amino acid sequence represent the N-terminal amino acid sequence obtained directly from purified human gastric lipase. Spaces in the directly obtained amino sequence represent undetermined amino acids. Amino acids -19 to -1 represent a putative signal sequence and +1 to 379, the amino acid sequence of the mature gene. Broken underlining indicates the potential glycosylation sequence.

Plasmid vectors for the expression of methionine human gastric lipase were constructed based on plasmid pMA91 (also known by the designation pMA3013) as described in the published European patent application EP-A2-0073653. These vectors contain the yeast phosphoglycerate kinase (PGK) promoter and the PGK gene 3' end flanking sequences sandwiching the methionine-human gastric lipase gene. A plasmid pMB1 (not shown) was constructed by insertion of BglII fragment containing the entire pre human gastric lipase gene. The plasmid pYC3 (Figure 6) was constructed by removal of a BglII to AccI fragment from pMB1 containing the 3' end of the lipase gene and ligated to the BglII to AccI fragment of the 5'

end of the gene obtained from pCML1 (described above). This was inserted into the BglII site of pMA3013 to form pYC3. The plasmid pYC3 was transformed into the diploid strain MD50 and the haploid MD40/4C and transformants grown up in nitrogen based medium as described in the published European patent application EP-A2-0073653.

Plasmid pMA91-DEX (the construction of which is described in Example 1) was digested under standard conditions with BamHI restriction enzyme. The small BamHI fragment carrying most of the DEX structural gene, was replaced by two DNA fragments isolated from pYC3. These fragments were a BglII-AccI DNA fragment, comprising the 5' end of the hGL gene, and an AccI-BamHI DNA fragment comprising the 3' end of the hGL gene, and the pGK transcriptional terminator, and were isolated by the method of Vogelstein & Guillespie (Proc. Natl. Acad. Sci USA 76 (1979) 615-619. The purified DNA fragments were ligated together under standard conditons, and transformed into E.coli DH1.

The fusion junction was as follows:



Plasmid DNA was isolated from transformants and analysed by restriction digestion, one plasmid comprising the correct DNA fragments was designated pMA91DexhGL.

Plasmid pMA91DexhGL was transformed in S. cerevisiae strain MD404C, and the synthesis and secretion

of hGL was analysed by (1), an agar plate activity assay, and antibody reaction, and (2), radioactive assay of hGL activity. MD404C pMA91DexhGL, was plated onto yeast nitrogen base minimal plates containing the lipid tributyrin (0.75%), and zones of clearing around colonies, caused by lipase action on the tributyrin emulsion, were detected (Figure 7a). The plates comprising zones of clearing were analysed for hGL protein, by overlaying with a nitro cellulose filter for 2 hours. The filter was then treated as described for Western Blots (Towbin, H., et al (1979), "Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Introcellulose Sheets: Procedure and some applications, PNAS" 76 : 4350-4353) using a polyclonal antibody to hGL, and iodinated protein A (Figure 7b). After washing, the filter was analysed by autoradiography, and immunoreactive material was detected, within the zones of clearing.

MD404C pMA91DexhGL was grown in liquid culture (100ml) at 30°C, in yeast nitrogen base minimal medium. The cells were harvested by centrifugation, and the medium fraction filtered through sterile filters. The cell pellet was divided into two halves, one was resuspended in 10mM Tris. HCl(pH7.5) buffer, and broken in a French pressure cell, to give total cell extracts. The other half of the pellet was subjected to lyticase treatment (J.H. Scott & R. Schekman J. Bact. 142 414-423, (1980) to generate cell wall, and cell cytoplasm fractions. These fractions were analysed for lipase activity as follows:

Stock Solutions

1. Label: 15μmoles phosphatidyl choline, 5mCi ^{3}H-triolein, 200μmoles cold triolein and 3.3ml anhydrous glycerol were sonicated for 2 periods of 1 min. on setting 7.
2. Cold emulsion as for 1 above without label.

3. Reaction buffer: 0.1M Na_2HPO_4 adusted to pH5.4 with 0.1M citric acid.
4. Extraction buffer: 0.2M Glycine/NaOH pH12.5.
5. Extraction solvent: Methanol: Chloroform: Hexane (1.41 : 1.25 : 1).

Method

1. Prepare a 7% w/v soluton of defatted BSA in the reaction buffer.
2. Prepare a dilution of label in cold emulsion : one in ten is suitable.
3. Add together equal quantities of the above two and sonicate to give a fresh substrate preparation. (Setting 7, 50% pulsed for 2 x 1 minute periods on ice).
4. Aliquot 50µl of substrate and 25µl BSA buffer per tube.
5. Equilibrate tubes at 37°C for 30 mins.
6. Add 25µl of lipase (dilutions in citrate buffer). Mix thoroughly.
7. Incubate with shaking 30 mins.
8. Add 1.625ml of extraction solvent to stop reaction and 0.525ml of alkaline buffer. Vortex vigorously.
9. Centrifuge to separate phases : 2000 RPM, 25°C, 15 mins. IEC Centra.
10. Count 0.5ml aliquots of the aqueous phase in 3.5ml Beckman Redisolve.

The results are shown in Table VI below:

TABLE VI

Fraction	% Total Lipase Activity	mg/L* hGLipase
Media	11	0.7
Cell Wall	21	1.3
Intra Cellular	68	4.2
Total Cellular	100	5.2

* These values were estimated using hGLipase enzyme standard.

Gastric lipase activity was detected in all the cell fractions assayed; approximately 32% of all the activity was extracellular (cell wall or medium) whilst 67% was intracellular. These results demonstrate that the 5' region of the DEX gene directs heterologous proteins to the medium and cell wall. Md404C carrying pYC3, a plasmid expressing met hGL, was used as a control, and only showed lipase activity in the intracellular fraction.

The protein product of the human gastric lipase - Dextrinase gene fusion was further analysed using Western blot analysis of 10% polyacrylamide - SDS gels (Towbin, H., et al (1979)). The results of such a blot are shown in Figure 8. Track 1 is the pattern of bands observed with a cell extract made from S. cerevisiae MD50 carrying pYC3 (Figure 6), the major immunoreactive band was the size expected for mature human gastric lipase (plus an additional methionine residue). Tracks 2, 3 and 4 are the pattern of bands observed with a cell extract made from MD50 carrying pMA91DexhGL. There is one protein band corresponding in size to the matrue hGL protein, this suggests that the fusion protein signal has been cleaved off. This process is indicative of entry into the endoplasmic reticulum and the secretory process. Above this band there are at least 4 other bands of immunoreactivity, which are not found in track 1, these bands are typical of protein glycosylation and secretion, and hGL is naturally glycosylated during secretion. These results therefore, provide further evidence that the dextrinase leader sequence is capable of directing heterologous proteins into the secretory process.

Example 3

An experiment was conducted to show that a DNA sequence containing the signal sequence of the yeast DEX gene (encoding amylo-α-1,4-glucosidase) can direct secretion

of human interferon ∝-2 from yeast cells into the culture medium.

The plasmid used for these experiments was constructed by replacing the DEX coding region in pMA91-DEX with a 940bp BamHI fragment containing the human interferon-∝-2 (IFN) gene. This gave plasmid pMA91-DEX-IFN, in which 110bp of the 5' region of DEX containing the signal sequence is linked to the IFN gene (Figure 9). In this plasmid, the hybrid DEX-IFN gene is under the control of the PGK promoter. pMA301-1 which contains the IFN gene also under control of the PGK promoter (Mellor et al, (1985) Gene, 33, 215-226) was used as a control in these experiments.

The yeast strain MD40/4c (ura2, trp1, leu2.3, leu2.12, his3.11, his3.15) was transformed with pMA91-DEX-IFN and pMA301-1 to produce transformants T91-DEX-IFN and T301-1 respectively. T91-DEX-IFN and T301-1 were grown overnight at 30°C in synthetic complete medium without leucine (SC: 0.67% yeast extract without amino acids, 1% glucose) to a cell density fo 4 x 10^6 cells/ml. The cells were harvested and soluble protein extracts were prepared as described by Mellor et al (1983, Gene. 24, 1-14). The protein extracts were analysed for the presence of IFN by Western blotting procedures using a monoclonal antibody from cell-line NK2 (Celltech Ltd.) as a probe. Cell extracts T91-DEX-IFN produced a polypeptide which had the mobility of authentic IFN as compared with T301-1 extracts and also a polypeptide of higher molecular weight which is thought to be a DEX-IFN fusion protein (data not shown).

To determine whether the DEX signal sequence could direct secretion of IFN into the culture medium, T91-DEX-IFN and T301-1 were grown at 30°C overnight in SC without leucine to a density of 3 x 10^7 cells/ml (early stationary phase). 150ml of each culture were centrifugated at 3K for 5 min. and the media supernatants,

to which 1mM PMSF (Sigma) was added, were dialysed against STE (10mM NaCl; 10mM Tris pH7.5; 1mM EDTA) for 4 days. The supernatant volumes were reduced by dialysing agaisnt PEG 4000 (Sigma) for approximately 2 hrs. followed by further dialysis against STE for 3 days. The supernatants were dried down in a Speed Vac Concentrator (Savant Instruments Inc.) and resuspended in a total volume of 400µl sterile water (AR grade). The samples were divided into two portions and both aliquots were assayed in parallel for IFN activity using an IRMA assay (supplied by Celltech Ltd.). The results are shown in Table VII.

TABLE VII

Assay for IFN Activity in Media Supernatants

IFN Standards Units /200µl	cpm Sample 1	Sample 2
1000	3111	3143
500	2083	2305
250	1022	921
100	502	561
50	334	258
25	180	163
12.5	104	127
0	60	59

Media Supernatants

	cpm Sample 1	Sample 2	[IFN] Units/sample
T301-1 undiluted	76	68	19
$^1/_{10}$ diluted	63	62	0
T91-DEX-IFN undiluted	3736	3707	1000 u
$^1/_{10}$ diluted	1863	2242	468

IFN was detected in the undiluted (off scale 1000 µ) and the $^1/_{10}$ diluted samples (mean = 468u) of media from T91-DEX-IFN. Very small amounts of IFN (mean = 19u) were detected in undiluted media samples from T301-1. Extrapolation of the IFN activity in diluted samples of media from T91-DEX-IFN gives a total IFN activity of 9.36 x 10^3 units from 150ml culture ; this is equivalent to 46.8ng IFN.

These results show that the DEX signal sequence can direct secretion of a heterologous gene product into the culture medium. The conditions for secretion could be optimised using yeast strains whose genetic backgrounds may be more compatible with secretion of the homologous DEX product than that of MD40/4c. Similarly, experiments need to be carried out to define the optimum growth conditions for most efficient secretion.

It will be understood that the invention is described by way of Example only and modifications of detail may be made within the scope of the invention.

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CLAIMS:

1. A precursor polypeptide having the amino acid sequence:

met-gln-arg-pro-phe-leu-leu-ala-tyr-leu-
val-leu-ser-leu-leu-phe-asn-ser-ala-leu-gly-X

wherein X is a polypeptide.

2. A DNA sequence coding for a precursor polypeptide having the amino acid sequence:

met-gln-arg-pro-phe-leu-leu-ala-tyr-leu-
val-leu-ser-leu-leu-phe-asn-ser-ala-leu-gly-X

wherein X is a polypeptide.

3. A DNA sequence according to claim 2 having the nucleotide sequence:

5' -ATGCAAGACCATTTCTACTCGCTTAT-
TTGGTCCTTTCGCTTCTATTTAAC-
TCAGCTTTGGGT(X^1)-3'

wherein X^1 is the nucleotide sequence coding for polypeptide X.

4. A eukaryotic expression vector including a DNA sequence according to claim 2 or 3 positioned relative to a promoter capable of directing expression of the DNA sequence when the vector is transformed into a eukaryotic host organism, provided that when the polypeptide X is amylo-α-1, 4-glucosidase, the promoter is not an amylo-α-1, 4 glucosidase gene promoter.

5. A eukaryotic expression vector according to claim 4 wherein the promoter is derived from at least a functionally active portion of the 5' region of the yeast phosphoglycerate kinase gene.

6. A eukaryotic host organism transformed with a vector according to claim 5.

7. A yeast transformed with a vector according to claim 6.

8. A brewing strain of yeast transformed with a vector according to claim 4 to 5 wherein the polypeptide X is an amylolytic enzyme.

9. A fermentation process for producing ethanol comprising the step of culturing a yeast in the presence of starch or dextrin, transformed with a vector according to claim 4 or 5 wherein the polypeptide X is an amylolytic enzyme.

10. A process for the production of a polypeptide comprising growing, in a culture medium, a eukaryotic host organism transformed with a vector as defined in claim 4 and isolating the polypeptide from the culture medium.

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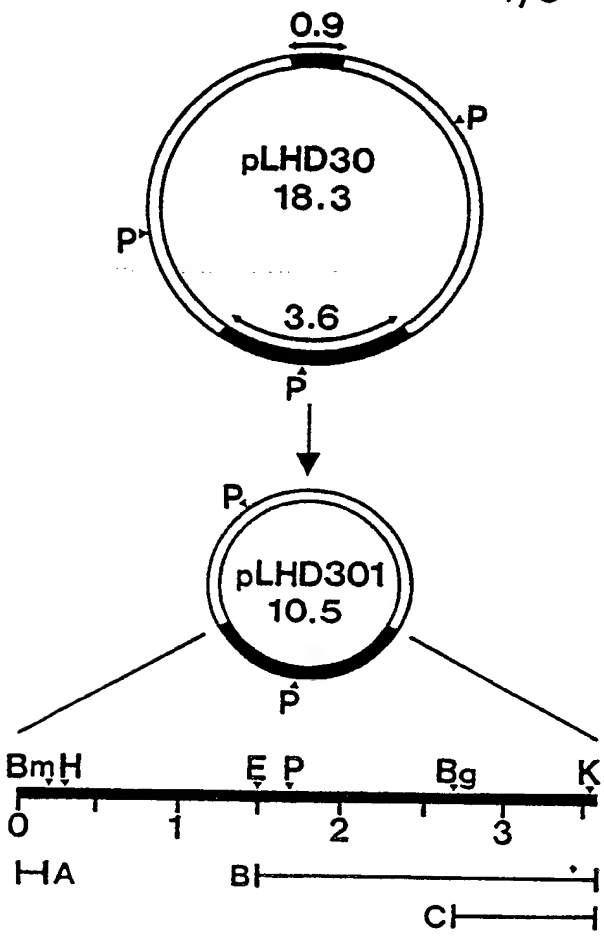
0.9
P
pLHD30
18.3
P
3.6
P
a.
P
pLHD301
10.5
P
b.
BmH
E P
Bg
K
0
1
2
3
HA
BI
CI
c.

FIG. 1

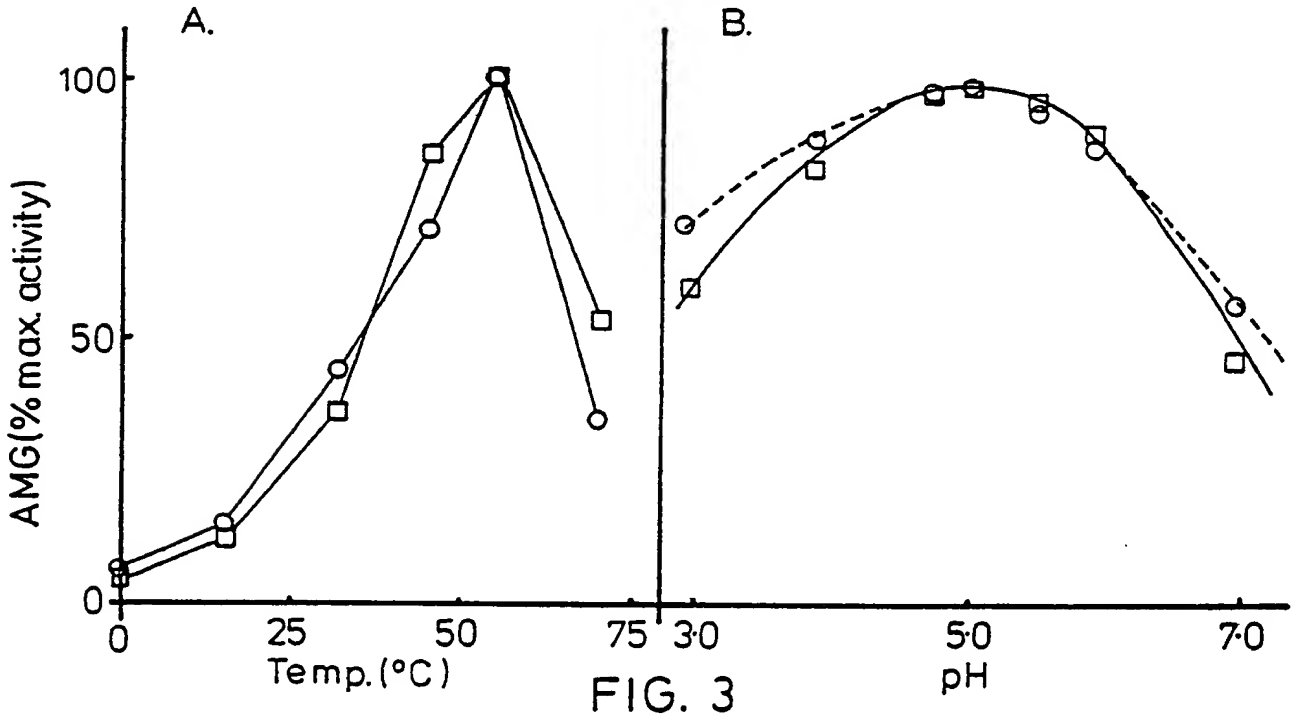
A.
B.
100
50
0
AMG(% max. activity)
0
25
50
75
Temp.(°C)
3·0
5·0
7·0
pH

FIG. 3

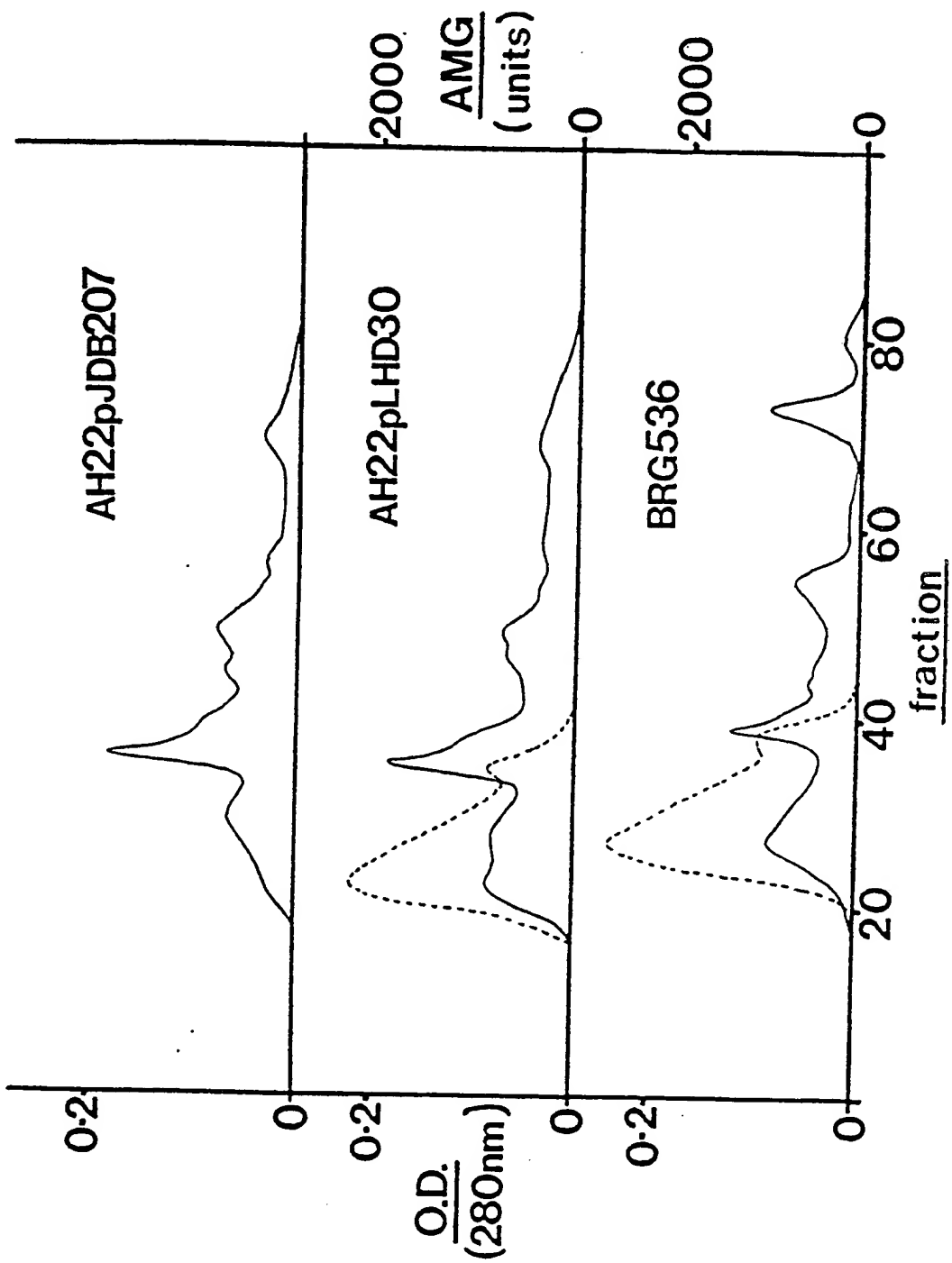
AH22pJDB207
AH22pLHD30
BRG536
AMG (units)
2000
0
2000
0
O.D. (280nm)
0·2
0
0·2
0
0·2
0
20
40
60
80
fraction

FIG. 2

FIG. 4 (i)

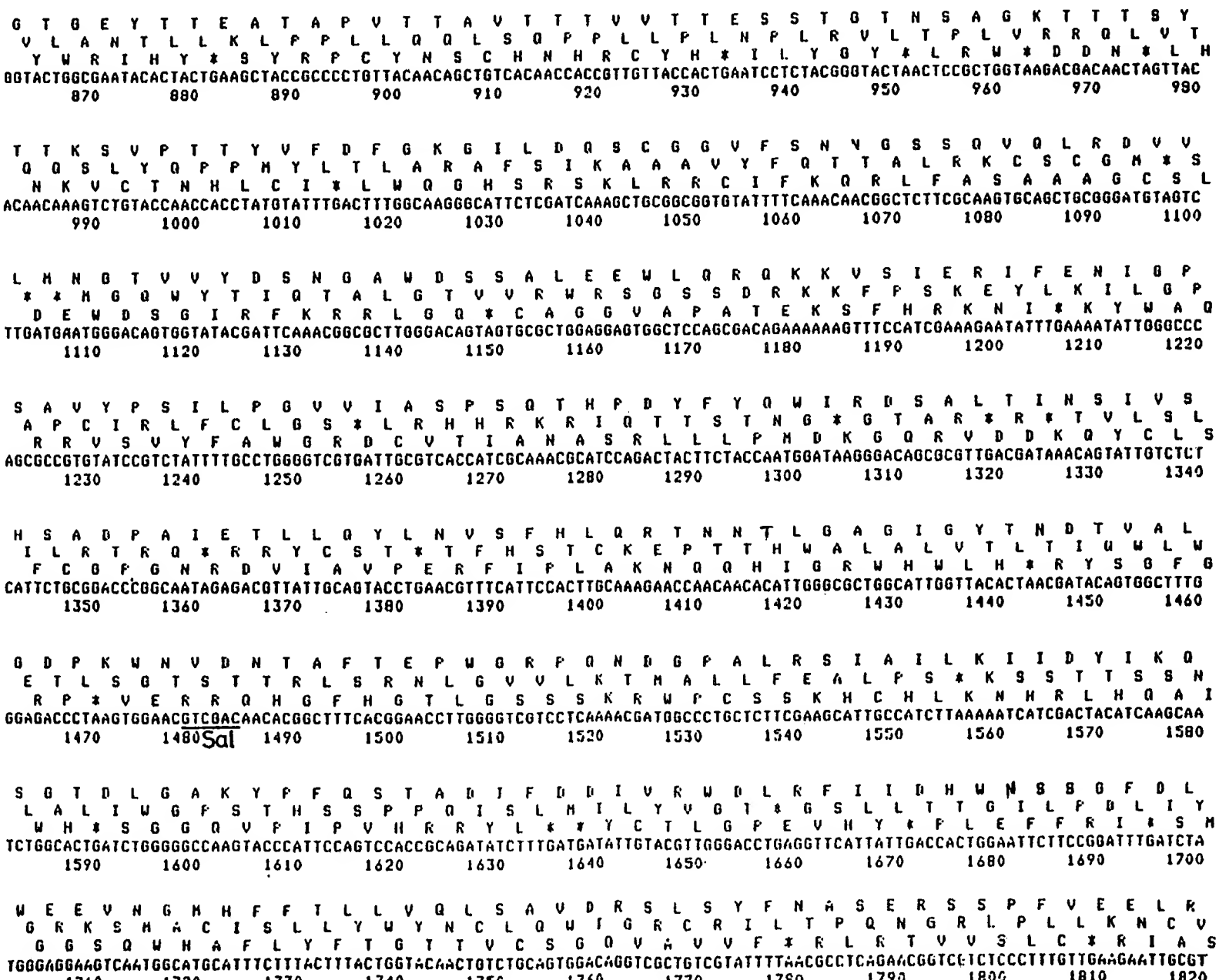

FIG. 4
(ii)

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Q T R R D I S K F L V D P A N G F I N Q K Y N Y I V E T P M I A D T L R S G L D
 R H A G T S F S F * W T L R M G L S T A S T I I L L R H P * L P T H * D P D W T
  D T P G H L Q V F S G P C E W V Y Q R Q V Q L Y C * D T H D C R H I E I R T G H
CAGACACGCCGGGACATCTCCAAGTTTTTAGTGGACCCTGCGAATGGGTTTATCAACGGCAAGTACAATTATATTGTTGAGACACCCATGATTGCCGACACATTGAGATCCGGACTGGAC
      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920      1930      1940

I S T L L A A N T V H D A P S A S H L P F D I N D P A V L N T L H H L M L H M R
 Y P L Y * L R T P S T M R H L L P I F R S I S M T L F S * T R C T I * C C T C V
  I H F I S C E H R P R C A I C F P S S V R Y Q * P C R P E H V A P F D V A H A F
ATATCCACTTTATTAGCTGCGAACACCGTCCACGATGCGCCATCTGCTTCCCATCTTCCGTTCGATATCAATGACCCTGCCGTCCTGAACACGTTGCACCATTTGATGTTGCACATGCGT
      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040      2050      2060

S I Y P I N D S S K N A T G I A L G R Y P E D V Y D G Y G V Q E G N P W V L A T
 R Y T P S T I A P K M Q R V L P W A G I L R T Y M M D M A L A R E I P G S W P R
  D I P H Q R * L Q K C N G Y C P G P V S * G R I * W I W R W R G K S L G P G M V
TCGATATACCCCATCAACGATAGCTCCAAAAATGCAACGGGTATTGCCCTGGGCCGGTATCCTGAGGACGTATATGATGGATATGGCGTTGGCGAGGGAAATCCCTGGGTCCTGGCCACG
      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160      2170      2180

C A A S T T L Y Q L I Y R M I S E Q M D L V V P M N N D C S N A F W S E L V F S
 V P L Q Q R F I S S F T D T S L S S M T W L S Q * T T I V R T M F G A S W Y S P
  C R F N N A L S A H L Q T H L * A A * L G C P N E Q R L F E R I L E R A G I L Q
TGTGCCGCTTCAACAACGCTTTATCAGCTCATTTACAGACACATCTCTGAGCAGCATGACTTGGTTGTCCCAATGAACAACGATTGTTCGAACGCATTTTGGAGCGAGCTGGTATTCTCC
      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280      2290      2300

N L T T L G N D E G Y L I L E F N T P A F N Q T I Q K I F Q L A D S F L V K L K
 T S R L W E M T K A I * F W S S I M L P S I K P Y K K S S N * L I M S W S S * K
  P M D F G K * R R L F D F G V Q Y T C L Q S N M T K N L P T S * F I L G Q A E S
AACCTCACGACTTTGGGAAATGACGAAGGCTATTTGATTTTGGAGTTCAATACACCTGCCTTCAATCAAACCATACAAAAAATCTTCCAACTAGCTGATTCATTCTTGGTCAAGCTGAAA
      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400      2410      2420

A M V G T D G E L S E Q F N K Y Y G F M Q G A Q M L T W S Y T S F W D A Y Q I R
 L T W E Q T G N * V N N L T N T Q G L C R V P N T L P G P I L H S G M P I K * D
  S R G N R R G T K * T I * Q I M R V Y A G C P T P Y L V L Y F I L G C L S N K T
GCTCACGTGGGAACAGACGGGGAACTAAGTGAACAATTTAACAAATACACAGGGTTTATGCAGGGTGCCCAACACCTTACCTGGTCCTATACTTCATTCTGGGATGCCTATCAAATAAGA
      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520      2530      2540

                Stop
Q E V L Q S L [*] T K K N K R K A R S I H K C I S * I F T S N I Y I Y T Y L Q N S
 K K F Y R V C R Q K K I K E K R E V Y T S V F F R Y L H Q I Y I Y I L I Y K T L
  R S F T E F V D K K K * K K S E K Y T Q V Y F L D I Y I K Y I Y I Y L F T K L *
CAAGAAGTTTTACAGAGTTTGTAGACAAAAAAAAAATAAAAGAAAAGCGAGAAGTATACACAAGTGTATTTCCTAGATATTTACATCAAATATATATATATACTTATTTACAAAACTCT
      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640      2650      2660

D I I N * L D N Y V G T S S P T T F G S S F H F L I L C Q L V A G L Y L S T
 I L * I N * I T M S E R P A Q F R L A V L F T F S S C V H L L P D C I C R
  Y Y K L I R * L C R N V Q P N H V W Q F F S L S H P V S I C C R I V S V D
GATATTATAAATTAATTAGATAACTATGTCGGAACGTCCAGCCCAACCACGTTTGGCAGTTCTTTTCACTTTCTCATCCTGTGTCAACTTGTTGCCGGATTGTATCTGTCGAC<---
      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760      2770         7
```

FIG. 4
(iii)

SUBSTITUTE SHEET

FIG. 5

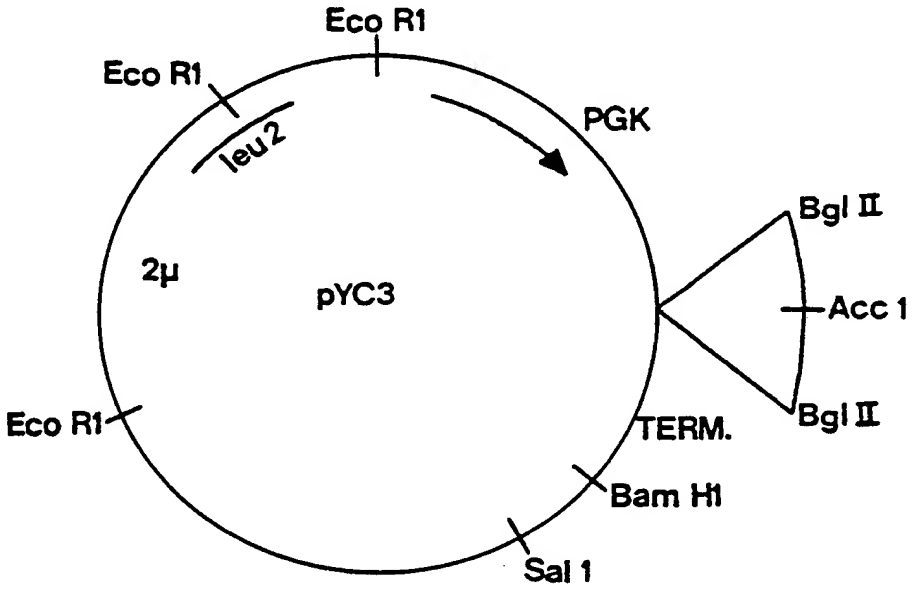
Eco R1
Eco R1
leu2
PGK
Bgl II
2μ
pYC3
Acc 1
Eco R1
TERM.
Bgl II
Bam H1
Sal 1

FIG. 6

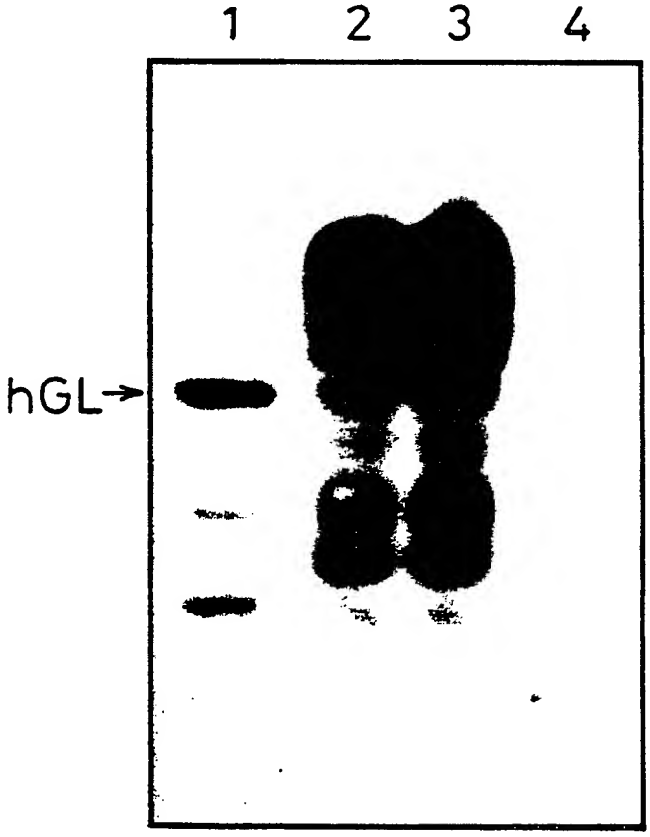
1
2
3
4
hGL→

FIG. 8

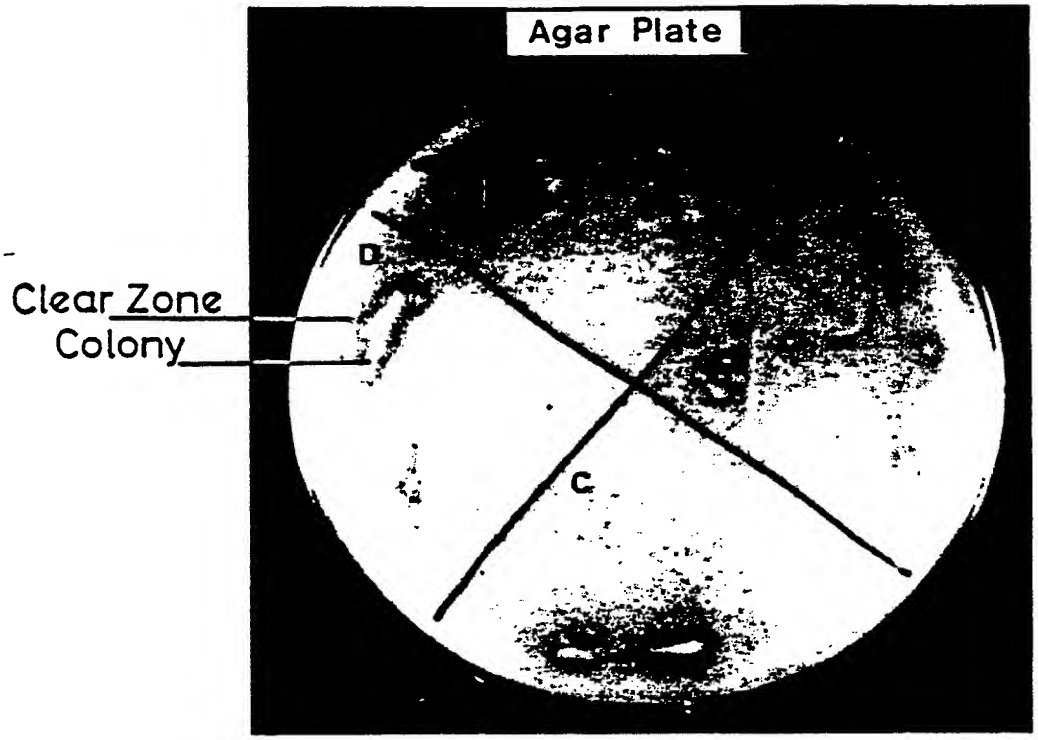
Agar Plate
Clear Zone
Colony
D
C

FIG. 7(a)

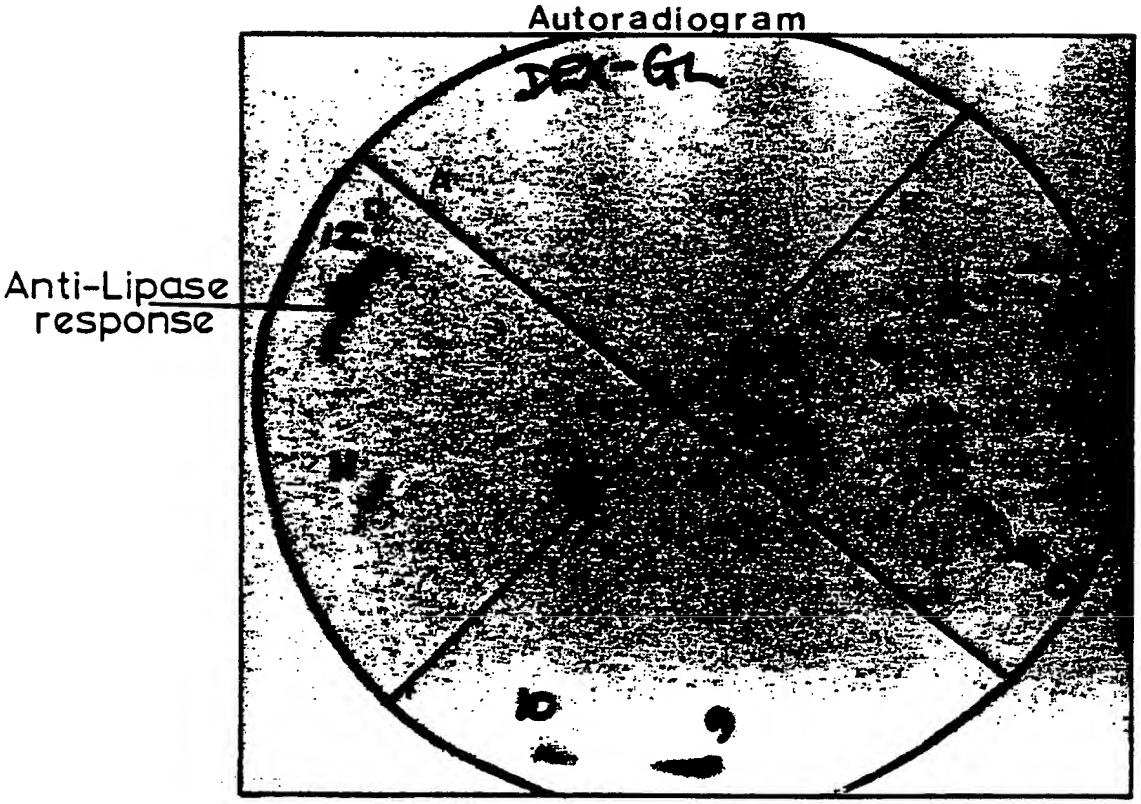
Autoradiogram
Anti-Lipase
response
A
B
C
D

FIG. 7(b)

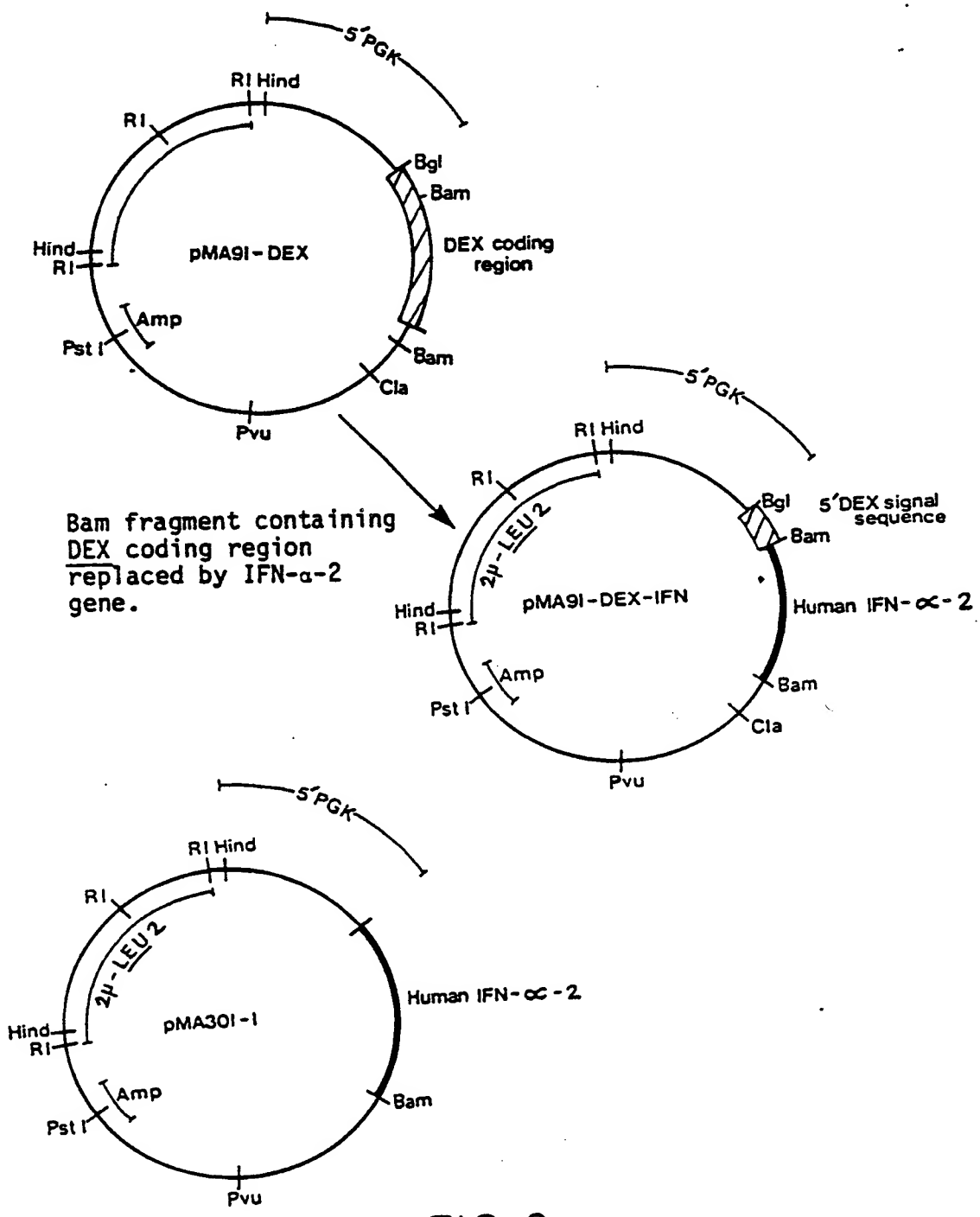
5'PGK
RI Hind
RI
Bgl
Bam
DEX coding region
Hind
RI
pMA9I-DEX
Amp
Pst I
Bam
Cla
Pvu
Bam fragment containing DEX coding region replaced by IFN-α-2 gene.
5'PGK
RI Hind
RI
2μ-LEU2
Bgl
5'DEX signal sequence
Bam
Hind
RI
pMA9I-DEX-IFN
Human IFN-α-2
Amp
Pst I
Bam
Cla
Pvu
5'PGK
RI Hind
RI
2μ-LEU2
Human IFN-α-2
Hind
RI
pMA30I-1
Amp
Pst I
Bam
Pvu

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00599

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 1/18; C 12 P 19/20; C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Nature, volume 308, no. 5960, 12 April 1984 S.J. Rothstein et al.:"Secretion of a wheat α-amylase expressed in yeast", pages 662-665, see figure 1	1-10
Y	EP, A, 0126206 (CETUS CORPORATION) 28 November 1984, see table II[B]; example 2	1-10
Y	Agricultural and Biological Chemistry, volume 47, no. 11, November 1983 I. Yamashita et al.:"Molecular cloning of a glycoamylase-producing gene in the yeast Saccharomyces", see the whole document	1-10
Y	Brewers' Guardian, September 1984, R.S. Tubb:"Genetic development of yeast strains", pages 34-37, see page 36, last paragraph; page 37 (cited in the application)	1-10
X,P	Journal of Bacteriology, volume 161, no. 2, February 1985,	./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th April 1986	23 AVR. 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

International Application No. PCT/GB 85/00599

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	I. Yamashita et al.:"Nucleotide sequence of the extra-cellular glucoamylase gene STA1 in the yeast Saccharomyces diastaticus", pages 567-573, see figure 1, aminoacids 12-32	1-3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00599 (SA 11727)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/04/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0126206	28/11/84	WO-A-	8402921	02/08/84
		AU-A-	2499384	15/08/84
		JP-A-	60070079	20/04/85